

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |  |    |   |
|--|--|----|---|
| <p>(51) International Patent Classification<sup>5</sup> :<br/>A23L 1/054, 1/30, A61K 47/00<br/>A23D 9/00, 9/02, 9/04<br/>A23K 1/00, 1/18, C12P 7/64<br/>C12P 7/40</p>  |  | A1 | <p>(11) International Publication Number: WO 91/11918<br/><br/>(43) International Publication Date: 22 August 1991 (22.08.91)</p> |
| <p>(21) International Application Number: PCT/US91/00733<br/><br/>(22) International Filing Date: 4 February 1991 (04.02.91)<br/><br/>(30) Priority data:<br/>479,135 13 February 1990 (13.02.90) US<br/><br/>(60) Parent Application or Grant<br/>(63) Related by Continuation<br/>US 479,135 (CIP)<br/>Filed on 13 February 1990 (13.02.90)<br/><br/>(71) Applicant (for all designated States except US): MARTEK<br/>CORPORATION [US/US]; 6480 Dobbin Road, Columbia,<br/>MD 21045 (US).</p>  |  |    |   |
| <p>(72) Inventors; and<br/>(75) Inventors/Applicants (for US only) : KYLE, David, John<br/>[CA/US]; 1801 Narberth Road, Catonsville, MD 21228<br/>(US). REEB, Sue, Ellen [US/US]; 3033 Hamilton Avenue,<br/>Baltimore, MD 21214 (US). SICOTTE, Valerie,<br/>Jacqueline [US/US]; 4302 B Alan Drive, Baltimore, MD<br/>21229 (US).<br/><br/>(74) Agents: FIGG, E., Anthony et al.; Rothwell, Figg, Ernst &amp;<br/>Kurz, 1700 K Street, N.W., #800, Washington, DC<br/>20006 (US).<br/><br/>(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.<br/><br/>Published<br/>With international search report.</p> |  |    |   |
| <p>(54) Title: DOCOSAHEXAENOIC ACID, METHODS FOR ITS PRODUCTION AND COMPOUNDS CONTAINING THE SAME</p>  |  |    |   |
| <p>(57) Abstract</p> <p>This invention relates to a process for producing a single cell edible oil containing DHA, Docosahexaenoic acid, to the oil itself and to uses for the oil. A marine microorganism, such as <i>Cryptothecodium cohnii</i>, is cultivated in fermentors and induced to produce the single cell oil which subsequently is recovered by extraction with solvents.</p>   |  |    |   |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |  |    |                          |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria                  | ES | Spain                                    | MG | Madagascar               |
| AU | Australia                | FI | Finland                                  | ML | Mali                     |
| BB | Barbados                 | FR | France                                   | MN | Mongolia                 |
| BE | Belgium                  | GA | Gabon                                    | MR | Mauritania               |
| BF | Burkina Faso             | GB | United Kingdom                           | MW | Malawi                   |
| BG | Bulgaria                 | GN | Guinea                                   | NL | Netherlands              |
| BJ | Benin                    | GR | Greece                                   | NO | Norway                   |
| BR | Brazil                   | HU | Hungary                                  | PL | Poland                   |
| CA | Canada                   | IT | Italy                                    | RO | Romania                  |
| CF | Central African Republic | JP | Japan                                    | SD | Sudan                    |
| CG | Congo                    | KP | Democratic People's Republic<br>of Korea | SE | Sweden                   |
| CH | Switzerland              | KR | Republic of Korea                        | SN | Senegal                  |
| CI | Côte d'Ivoire            | LI | Liechtenstein                            | SU | Soviet Union             |
| CM | Cameroon                 | LK | Sri Lanka                                | TD | Chad                     |
| CS | Czechoslovakia           | LU | Luxembourg                               | TG | Togo                     |
| DE | Germany                  | MC | Monaco                                   | US | United States of America |
| DK | Denmark                  |    |  |    |                          |

DOCOSAHEXAENOIC ACID, METHODS FOR ITS  
PRODUCTION AND COMPOUNDS CONTAINING THE SAME

Background of the Invention

This invention relates to edible, single-cell oil  
5 containing docosahexaenoic acid (DHA). The invention  
also relates to methods of producing such oil  
containing DHA in commercially viable yields and to  
products containing the oil.

DHA is an omega-3-fatty acid and is the most  
10 abundant long chain polyunsaturated fatty acid (PUFA)  
in the grey matter of the brain. Omega-3-fatty acids  
in general are known to be beneficial in reducing the  
incidence of coronary heart disease [Lands, Fish and  
Human Health (1986) Academic Press]. However, the  
15 metabolism of omega-3-fatty acids is not well  
understood. Thus, precise clinical dosages and  
efficacy remain unknown.

Cold water marine fish are a known source of  
omega-3-fatty acids, including DHA. U.S. Patent No.  
20 4,670,285 discloses the use of fish oil from fish such  
as menhaden and herring as a source of C<sub>22</sub> omega-3-fatty  
acids. Indeed, fish oils are the primary commercial  
source of omega-3-fatty acids. Often, however, fish  
oils are unusable for human consumption because of  
25 contamination with environmental pollutants such as  
PCB's.

There also are problems associated with the recovery of fish oils containing DHA for food uses. Such oils often have a fishy odor and unpleasant tastes associated with the oxidation products of the fatty acids. These tastes and toxicities of peroxides render the oils unsatisfactory for use in edible compositions such as baby food and infant formulas.

Marine microorganisms also are known to contain DHA. In particular, various species of dinoflagellates are known to contain DHA. Harrington et al., "The Polyunsaturated Fatty Acids of Marine Dinoflagellates" J. Protozoal., 17:213-219 (1970), characterize the fatty acid content of eight photosynthetic and one heterotrophic marine dinoflagellates, and conclude that the dinoflagellates are a primary producer group of docosahexaenoic acid and contribute substantial amounts of that compound to the marine food chain.

Successful cultivation of dinoflagellates to produce an edible oil containing DHA has not been achieved. Dinoflagellates in general are very slow growing and are shear sensitive. Guillard et al., Dinoflagellates, (1984) Academic Press. The prior art discloses that even a small amount of agitation in the culturing vessel reduces growth of the cultures. However, such agitation would be necessary to achieve adequate oxygenation in order to maximize growth for commercial production.

DHA is thought to be essential for the proper brain and vision development of infants because, as noted above, it is the most abundant long chain PUFA in the brain and retina. Although a metabolic pathway exists in mammals for the biosynthesis of DHA from dietary linolenic acid, this pathway is bioenergetically unfavorable [Crawford, P. AOCS. Short

Course in Polyunsaturated Fatty Acids and Eicosanoids, pp. 270-295 (1987)] and mammals, like fish, are thought to obtain most of their DHA from dietary sources. In the case of infants, the most likely source would be 5 human milk. Indeed, DHA is the most abundant C20 omega-3 PUFA in human milk. Generally, however, DHA is absent from infant formulas. U.S. Patent No. 4,670,285 does disclose an infant formula containing omega-3-fatty acids. However, the acids utilized therein are 10 obtained from egg or fish (Talapia) oil and have associated therewith the unpleasant characteristics previously described. Furthermore, fish oils generally contain another omega-3-fatty acid, eicosapentaenoic acid (EPA), an undesirable component in infant formulas 15 because of its prolonged anticoagulant effects and its depression of arachidonic levels in infants. This has been correlated with reduced rates of infant weight gain (Carleson et al. INFORM 1:306.) Indeed, EPA levels are very low in human milk (less than one-fourth 20 that of DHA).

Accordingly, it is an object of the present invention to provide a single-cell edible oil containing DHA. Preferably this oil will have no significant quantities of other polyunsaturated fatty acids (PUFA's), i.e. greater than about 2% of the total fatty acid content. In general, it is an object of the 25 present invention to produce single-cell oil in commercially viable yields. The oil, characterized herein as a "designer" oil, after extraction can be used in infant formulas, baby foods, dietary 30 supplements and pharmaceuticals.

In addition, it would be desirable to acquire further knowledge of the metabolic pathway of omega-3-fatty acids. Isotopically labeled DHA would be of

great utility in this regard. However, to date, no method has been known to produce abundant quantities of isotopically labeled DHA. Thus, it also is an object of the present invention to provide isotopically labeled DHA in sufficient quantities to undertake such research.

#### Summary of the Invention

The present invention relates to the cultivation of microorganisms, notably dinoflagellates, in a fermentor, induction of those microorganisms to produce significant quantities of single cell oil containing a high proportion of DHA and recovery of that oil. As used herein, "single cell oil" refers to a lipid product of a unicellular organism. The present invention also includes mutant organisms capable of producing enhanced quantities of single-cell oil containing at least about 20% by weight DHA and includes single cell oil containing DHA.

The present invention provides an economical method of obtaining enhanced levels of edible oils containing DHA. Additionally, the method permits the commercial cultivation of dinoflagellates in elevated cell densities.

Edible oils produced by the method of this invention lack unpleasant tastes and fishy odors and also are free of environmental contaminants often found in DHA-containing oils from conventional sources. Accordingly, the present invention further includes food products containing the oil of this invention.

Brief Description of the Drawings

Figures 1, 2 and 3 are graphic illustrations of C. cohnii biomass accumulation over time with the addition of various nutrients.

5

Detailed Description of the Best Mode  
Of Practicing the Invention

In accordance with the present invention, microorganisms capable of producing a single cell oil containing DHA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably the single cell oil will contain at least about 20% by weight DHA.

Any microorganisms capable of producing a single-cell edible oil containing DHA can be used in the present invention. For example, photosynthetic diatoms can be used. Preferred microorganisms are marine dinoflagellates, including Cryptocodinium sp. Especially preferred is Cryptocodinium cohnii, an obligate heterotroph requiring a reduced carbon source for growth. C. cohnii is preferred because it contains a fatty acid profile in which DHA is the only PUFA present in sufficient quantities (greater than about 1% of the total amount of PUFAs). Samples of this organism, designated MK8840, have been deposited with the American Type Culture Collection at Rockville, Maryland, and assigned accession number 40750. As used herein, microorganism, or any specific type of microorganism, includes wild strains, mutants or recombinant types. Any microorganism which produces enhanced levels of oil containing DHA is considered to be within the scope of this invention. One of the features of the present invention is its recognition of the edible oil-producing capability of microorganisms

such as dinoflagellates and the attendant solution to the problem of maintaining a reliable, economic source of such oils. Accordingly, wild-type and recombinant microorganisms designed to produce single cell oil containing DHA are an aspect of this invention. Such recombinant organisms would include those designed to produce greater quantities of DHA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Also included would be microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of single cell oil containing DHA as the comparable wild-type microorganism.

In general, those of skill in the art would not consider C. cohnii a suitable organism for cultivation in a fermentor. Previous workers have commented on the extremely complex mixture of nutrients required to successfully cultivate C. cohnii. Gold et al. Protozoal, 13:255-257 (1966); Guillard, et al. in "Dinoflagellates", Academic Press (1984); Henderson, et al., Phytochemistry 27:1679-1683 (1988). In contrast, the present invention achieves the cultivation of DHA-producing microorganisms in a simple medium containing glucose and yeast extract. Use of these components in a solution such as seawater provides economically significant growth rates and cell densities. For example, during the course of a 3-5 day fermentation, C. cohnii cell densities of at least 10 grams of biomass per liter of solution, and typically from 20 to about 40 grams per liter, can be attained. Such densities have not heretofore been attainable.

Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor (STF) or in an air lift fermentor (ALF), both types known to those of skill in the art. When a STF is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and sparging renew the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep the tip speed at not greater than about 500 cm/sec, preferably not greater than about 300 cm/sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear is within the purview of those of skill in the art. The use of such strains is expressly included in this invention.

As noted above, seawater is an acceptable medium for the nutrient solution. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted to reduced salinities, such as 1/2 to 1/4 normal strength, with tap water or concentrated to 2 times normal strength. A preferred example is Instant Ocean® (IO) brand artificial seawater.

Although C. cohnii is a marine microorganism, some growth has been observed in zero salinity. The use of variants which grow well in reduced salinities is specifically encompassed by this invention.

Micronutrients can be added and may be required at low salinities. However, such micronutrients are known to those of skill in the art and generally are present in seawater or tap water. If the organism selected is

heterotrophic, such as C. cohnii, then a carbon source is added.

Preferably, after addition of the seawater medium to the fermentor, the fermentor containing the medium  
5 is sterilized and cooled prior to adding the nutrients and a seeding population of microorganism. (Although it is acceptable to sterilize the nutrients together with the seawater, sterilization in this manner can result in a loss of available glucose.) The nutrients  
10 and microorganism can be added simultaneously or sequentially.

An effective seed concentration can be determined by those of skill in the art. When a STF is used, the addition of a population of from about .05 to 1.0 grams  
15 of dry weight equivalent per liter at the beginning of the fermentation is preferred. This is about  $10^6$  cells per ml. Thus, for a 30 liter fermentor, 1-3 liters of seeding media, containing viable cells at a density of 20g dry weight per liter would be added.

Oxygen levels preferably are maintained at a D.O.  
20 of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly,  
higher yields of DHA require D.O. levels at from about  
10% to 50% of air saturation levels. Agitation tip  
25 speeds of 150-200 cm/sec in combination with an aeration rate of 1 VVM (volume of air/volume of fermentor per minute) provides D.O. levels of from about 20% to about 30% at biomass densities of about 25 g dry weight/liter of culture. Higher cell densities  
30 may require higher D.O. levels, which can be attained by increased aeration rates by O<sub>2</sub> sparging, or by increasing the air pressure in the fermentor.

Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided

to C. cohnii in the form of glucose. Other heterotrophs can use other reduced carbon sources, a matter easily determined by those of skill in the art, and autotrophs utilize carbon dioxide. C. cohnii will 5 also grow on other reduced, more complex, carbon sources. Typically, a fermentation is initiated with about 10-50 g/liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 50 to 150 g, preferably 50 to 100g 10 glucose/liter initially can be added, thereby minimizing the frequency of future additions. The amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

In addition to a reduced carbon source, a nitrogen 15 source, such as yeast extract (YE), is provided to the medium. Commercially available yeast extract is acceptable. For example, DIFCO or MARCOR brand yeast extract can be used. The yeast extract is an organic nitrogen source also containing micronutrients. Other 20 organic nitrogen sources easily can be determined by those of skill in the art. However, such compounds are generally more expensive than yeast extract. The use of variants capable of growing on urea or nitrates is within the scope of this invention. Typically, the 25 fermentation is initiated with about 6-12 g YE/liter. More YE can be added as required. A typical fermentation run requires from about 8 to 15 g YE/liter over the course of the run. Accordingly, that amount of YE can be added initially with a reduced need for 30 further additions. The precise amount can be determined by those of skill in the art. Generally, the ratio of glucose to YE is from about 2:1 to about 15:1.

The cultivation can be carried out at any life-sustaining temperature. Generally C. cohnii will grow at temperatures ranging from about 15°C to 34°C. Preferably the temperature is maintained at about 20-  
5 30°C. Strains which grow at higher temperatures are preferred, because they will have a faster doubling time, thereby reducing the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.

10 The cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 6.0 to about 7.0 is used for the growth phase. A base, such as KOH or NaOH, is used to adjust the media pH prior to inoculation. During  
15 the later stages of the fermentation, the culture medium tends to become alkaline. If desired, inorganic acid pH controls can be used to correct alkalinity during the growth phase.

20 Production of the single cell oil is induced in the dinoflagellates by the imposition of a stationary phase (i.e., by nitrogen depletion or a pH rise). YE deficiencies are caused by providing YE in a limiting amount such that the medium runs out of YE while available glucose remains. The present invention  
25 recognizes that it is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and YE as exemplary, a preferred ratio of carbon source to nitrogen source is about 10-15 parts glucose to 1 part  
30 YE. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.

After induction of oil production, the culture is grown for about 24 additional hours. During this period of oleosynthesis, the single cell oil containing

DHA is being synthesized and visible oil droplets become apparent. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the 5 added amount of YE. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general, the C. cohnii are cultivated for a time sufficient to produce single cell oil, usually from about 60 to about 90 hours, although this 10 time is subject to variation.

From about 15 to 30% of the resultant biomass, using wild-type C. cohnii, comprises extractable oil. Strain selection can increase this percentage and such selection is within the scope of this invention. 15 Preferably, the oil comprises greater than about 70% triglycerides having, in general, the following fatty acid composition.

20           15-20% myristic acid ( $C_{14:0}$ )  
          20-25% palmitic acid ( $C_{16:0}$ )  
          10-15% oleic acid ( $C_{18:1}$ )  
          30-40% DHA ( $C_{22:6}$ )  
          0-10% others

(Other oil components including polar lipids, such as phosphatidyl choline, also may be enriched in DNA.) 25 The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight and most preferably at least about 35% DHA by weight.

30 The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can

be determined by those of skill in the art. However, preferred solvents include pure hexane and supercritical fluids, such as supercritical CO<sub>2</sub>.

Extraction techniques using supercritical fluids are known to those of skill in the art and described in 5 McHugh et al., Supercritical Fluid Extraction, Butterworth, 1986. If the extraction solvent is hexane, a suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. 10 The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 20-50°C for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. Alternatively, a wet biomass paste (30-35% 15 solids) can be extracted directly with more polar solvents, such as ethanol, isopropanol or hexane/isopropanol mixtures. The residual biomass, i.e. the single cell edible oil extracted biomass of the microorganisms, such as C. cohnii, can be used as 20 an animal feed, containing as it does about 35-40% protein, 8-10% ash and 45-50% carbohydrates. Because of this high protein content and the elevated levels of DHA, the whole biomass paste can be used for aquaculture (e.g., shrimp, oysters, fish) feed.

25 The solvent then is removed from the oil by distillation techniques known to those of skill in the art. Conventional oilseed processing equipment is suitable to perform the filtering, separation and distillation. Additional processing steps, known to 30 those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and allow the separation of DHA-enriched polar lipid fractions.

Isotopically labeled single cell oils, including labeled DHA, can be easily obtained in sufficient quantities to permit research into the metabolic pathways of DHA by the method of this invention. When 5  $^{13}\text{C}$ -glucose or  $^{14}\text{C}$ -glucose is provided as the reduced carbon substrate, labeled DHA results.

The present invention also includes food products, such as infant formulas and baby foods, as well as dietary supplements, which contain the single-cell oil 10 containing DHA of the present invention. While those of skill in the art have recognized that infant formulas containing DHA are desirable, the prior art infant formulas contained DHA from fish oil, with its attendant unpleasant tastes and organoleptic 15 characteristics. Furthermore, fish oil supplementation of infant formula includes the addition of eicosapentaenoic acid (EPA), an omega-3-fatty acid known to possess anticoagulant activity and possibly responsible for reduction of arachidonic acid 20 biosynthesis. Such an activity is not desirable in infant formula or baby food and the single cell oil described herein contains no significant quantity of EPA. Food products, such as infant formula, containing the single cell oil of the present invention do not 25 have the unpleasant organoleptic characteristics of fish oil. The food products thus are more readily accepted by infants and adults alike. Preferably the infant formula of the present invention contains about 0.05% by weight of single cell oil containing DHA. The 30 baby food of the present invention, having a more solid constitution, preferably contains about 0.5% by weight of single cell oil containing DHA. In both instances, most preferably, the oil contains at least about 35% DHA.

The present invention includes pharmaceutical products including single cell oil containing DHA. Preferably the products contain at least about 35% DHA. Exemplary of such pharmaceutical products is one 5 suitable for use in providing total parenteral nutrition (TPN) to infants or adults. Additionally, dietary supplements containing the single cell oil are encompassed. Preferably, such supplements are in the form of gelatin capsules encapsulating said oil and may 10 be appropriate for pregnant women or breast feeding mothers. This especially may be true for such women who are vegetarians and do not get sufficient amounts of DHA in their diets.

The present invention also includes single cell oil containing DHA. Preferably the single cell oil contains at least about 20% by weight DHA. Most preferably the oil contains at least about 35% by weight DHA.

20 The present invention having been generally described, reference is had to the following non-limiting specific examples.

Example 1

25 Into a 30-liter working volume STF was loaded a medium of one half strength artificial seawater. Six liters of IO were combined with 18 liters of tap water. The fermentor containing the medium was sterilized and cooled to 28°C. Four hundred ml of concentrated YE (455g/l), 900 ml of glucose syrup (400 g/l) and one liter of inoculum from a seed fermentor containing 30 about  $2 \times 10^7$  cells/ml or a biomass of 20 g/liter (yielding a final concentration of about  $7 \times 10^6$  cells/ml or a biomass of about 700 mg/liter), were added to the medium. Agitation was set at 120 cm/sec

tip speed and aeration was set at 1 VVM (30 liters per minute). Additional glucose syrup (900 ml) was added after 30 hours and another 4.2 liters over the next 42 hours. Thus 6 liters of glucose syrup were added in total. Concentrated YE solution (400 ml) was added at hour 6 and another 1.2 liters were added over the next 48 hours until a total of 2.0 liters had been added. To maintain the D.O. at greater than 20%, at 24 hours the agitation tip speed was increased to 150 cm/sec and at 48 hours to 160 cm/sec. At 72 hours, the tip speed was increased to 200 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culturing conditions are depicted graphically in Figure 1. The culture was then harvested by centrifugation with the cell pellet retained. The harvested pellet of cells was frozen and dried (lyophilized) to about a 4% moisture content. Hexane (2.8 liters) was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50°C. A rotary evaporator was used to remove the hexane, producing about 175 g of crude DHA-containing oil.

#### Example 2

Into a 350-liter working volume STF was loaded a medium of one half strength artificial seawater made by combining 4.3 kg. of I.O.® with 230 liters of tap water. The fermenter containing the medium was sterilized and cooled to 28°C. 6.8 liters of concentrated YE (400g/l), 12.5 liters of glucose syrup (400g/l) and 30 liters of C. cohnii inoculum from a seed fermenter ( $10^6$  cells/ml or a biomass density of about 1.3g/liter) were added to the medium. Agitation was set at 73 cm/sec tip speed and aeration was set at

1 VVM (280 liters per minute). Additional glucose syrup (12 liters) was added after about 44 hours and another 43 liters over the next 32 hours. Thus, 67.5 liters of glucose syrup were added in total. The 5 glucose additions and the cell growth are depicted graphically in Figure 2.

To maintain the D.O. at greater than 20%, at 44 hours the agitation tip speed was increased to 175 cm/sec and at 55 hours to 225 cm/sec. At 76 hours, the 10 tip speed was decreased to 150 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture then was harvested. The harvested cells were dried to about a 4% moisture content. 15 Hexane was added to the dried biomass and stirred in a glass kettle for 2 hours at 25°C. A rotary evaporator was used to remove the hexane, producing about 700 g of crude DHA-containing oil.

### Example 3

20 Into a 30-liter working volume STF was loaded a medium of full strength artificial seawater made by combining 565g of I.O.® with 15 liters of tap water. The fermenter containing the medium was sterilized and cooled to 28°C. Four hundred ml of concentrated YE 25 (400g/l), 1.9 liters of glucose syrup (400g/l) and 1 liter of C. cohnii inoculum from a seed fermenter ( $10^6$  cells/ml or a biomass of about 2.0g/liter) were added to the medium. Agitation was set at 80 cm/sec tip speed and aeration was set at 1 VVM (20 liters per 30 minute). Additional glucose syrup (1.5 l) was added after 94 hours and another 1.1 liters at 116 hours. Thus 4.5 liters of glucose syrup were added in total.

To maintain the D.O. at greater than 20%, at 52 hours the agitation tip speed was increased to 160 cm/sec. At 66 hours, stationary phase was induced and in order to accomplish this, the pH was spiked with 4N KOH to 5 7.0 and the agitation tip speed was not further increased for the duration of the run. As shown in Figure 3, the culture was permitted to grow for an additional time sufficient to convert the final charge 10 of glucose into cellular oil. The culture then was harvested. The harvested cells were dried to about a 4% moisture content. Hexane was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50°C. A rotary evaporator was used to remove the hexane, producing about 65 g of crude DHA-containing 15 oil.

We claim:

1. A method of producing a single cell edible oil containing DHA comprising:

cultivating a microorganism capable of producing said single cell oil in a fermentor containing a nutrient solution having a limiting nutrient,

inducing said microorganism to produce said single cell oil by depleting said limiting nutrient, and

10 recovering said single cell oil.

2. The method of claim 1, wherein the microorganism is a dinoflagellate.

3. The method of claim 2, wherein the dinoflagellate is of the genus Cryptocodinium sp.

4. The method of claim 3, wherein said microorganism comprises Cryptocodinium cohnii.

5. The method of claim 4, wherein said nutrient solution comprises seawater.

6. The method of claim 5, wherein said seawater comprises an artificial seawater.

7. The method of claim 6, wherein said nutrient solution comprises a reduced salinity artificial seawater.

8. The method of claim 5, wherein said nutrient solution further comprises a reduced carbon source.

9. The method of claim 8, wherein said reduced carbon source comprises glucose.

10. The method of claim 5, wherein said limiting nutrient comprises an organic nitrogen source.

11. The method of claim 10, wherein said nitrogen source comprises yeast extract.

12. The process of claim 11, wherein the ratio of said glucose to said yeast extract is from about 2 to 15 parts glucose to 1 part yeast extract.

13. The method of claim 12, further comprising maintaining a dissolved oxygen content at a range of from about 10% to about 50% of air saturation.

14. The method of claim 13, wherein said range is from about 20-30%.

15. The method of claim 14, wherein said fermentor is a stirred tank fermentor or air lift fermentor.

16. The method of claim 15, wherein said stirred tank fermentor agitates said nutrient solution, thereby providing oxygen to said microorganisms.

17. The method of claim 16, further comprising aerating said nutrient solution.

18. The method of claim 17, wherein said aeration is at a rate of 1 volume of air per volume of fermentor per minute.

19. The method of claim 1, wherein said nutrient solution comprises  $^{13}\text{C}$ -glucose or  $^{14}\text{C}$ -glucose.

20. The method of claim 1, wherein the microorganism is cultivated to a density of at least about 10 g dry weight/liter of nutrient solution.

21. The method of claim 20, wherein the microorganism is cultivated to a density of at least about 20 g dry weight/liter of nutrient solution.

22. The method of claim 1, wherein at least about 15% of the mass of the cultivated microorganism comprises an extractable oil containing DHA.

23. The method of claim 22, wherein at least about 20% of the oil comprises DHA.

24. A method for the production of a single cell-edible oil containing DHA and lacking a fishy taste comprising:

- a) adding to a fermentor initially containing a nutrient solution comprising about one-half strength artificial seawater, 1-8% glucose and 0.4-0.8% yeast extract, about 0.5-1.0 g dry wt/liter (about  $10^6$  cells/ml) of C. cohnii,
- b) cultivating said C. cohnii at a temperature of from about 15°C to about 34°C and a pH of from about 5.0 to 9.0,
- c) incrementally adding glucose and yeast extract to said nutrient solution for about 56 hours,
- d) adding additional glucose to said nutrient solution for about 16 additional hours to induce said C. cohnii to produce a single cell edible oil,
- e) maintaining a dissolved oxygen content of about 20-30% throughout said cultivation, and
- f) harvesting said C. cohnii after about 60 to 90 hours.

25. The process of claim 24, further comprising extracting said single cell edible oil from said C. cohnii.

26. The process of claim 25, wherein said extraction is by treatment with a solvent such that said oil is extracted into said solvent.

27. The process of claim 26, wherein said solvent comprises hexane, ethanol, isopropanol, hexane/isopropanol mixtures or a supercritical fluid.

28. The process of claim 27, wherein said hexane is applied to said C. cohnii at a rate of about 4 liters per kilogram of dry biomass of said C. cohnii.

5 and is mixed with said biomass in a stirred reaction vessel for about 1 hour at about 20°C.

29. The process of claim 28, further comprising filtering said hexane containing extracted oil from said biomass and then removing said hexane by distillation from said oil.

30. A single cell-edible oil comprising docosahexaenoic acid.

31. The oil of claim 30, wherein said DHA comprises at least about 15% of the weight of said oil.

32. The oil of claim 31, wherein said DHA comprises at least about 30% of said weight.

33. An animal feed comprising the single cell edible-oil extracted biomass of microorganisms cultivated in accordance with the method of claim 1.

34. The animal feed of claim 33, wherein said microorganism comprises C. cohnii.

35. An aquaculture feed comprising cells of a microorganism capable of producing an edible-DHA-containing single cell oil cultivated under oil-producing conditions.

36. The feed of claim 35, wherein the aquaculture comprises shrimp, oysters or fish.

37. The feed of claim 35, wherein the microorganism is a dinoflagellate.

38. The feed of claim 37, wherein the dinoflagellate is C. cohnii.

39. An infant formula comprising a single cell edible oil containing DHA.

40. The infant formula of claim 39, wherein said oil comprises about 0.05% by weight (50 mg/100 ml) of said formula.

41. The infant formula of claim 40, wherein said oil comprises at least about 30% DHA.

42. Baby food comprising a single cell edible oil containing DHA.

43. The baby food of claim 42, wherein said oil comprises about 0.5% by weight of said food.

44. The baby food of claim 43, wherein said oil comprises at least about 35% DHA.

45. Dietary supplements containing the edible oil produced by the process of claim 1.

46. Infant formula containing the edible oil produced by the process of claim 1.

47. Baby food containing the edible oil produced by the process of claim 1.

48. Pharmaceutical products containing the single cell edible oil containing DHA produced by claim 1 and 24.

49. Pharmaceutical products containing the single cell oil of claim 30.

50. The pharmaceutical product of claim 49, wherein said product is useful for total parenteral nutrition.

51. Infant formula containing the single cell oil of claim 30.

52. Baby food containing the single cell oil of claim 30.

53. Dietary supplements containing the single cell oil of claim 30.

54. The dietary supplement of claim 53, wherein said supplement comprises a gelatin capsule containing said oil.

55. A recombinant microorganism which produces single cell oil containing DHA.

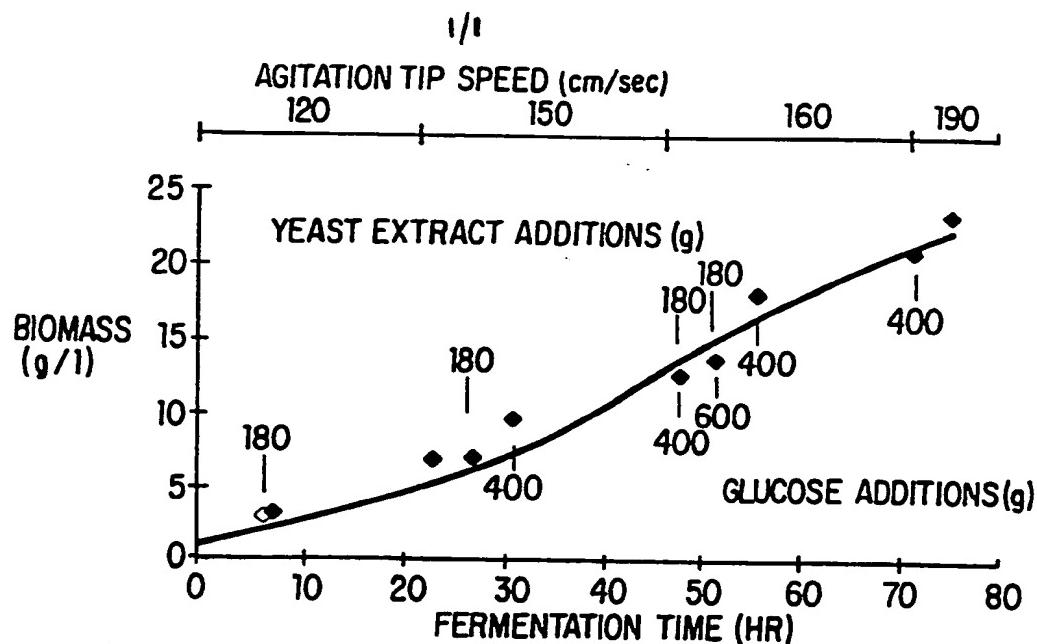


FIG. 1

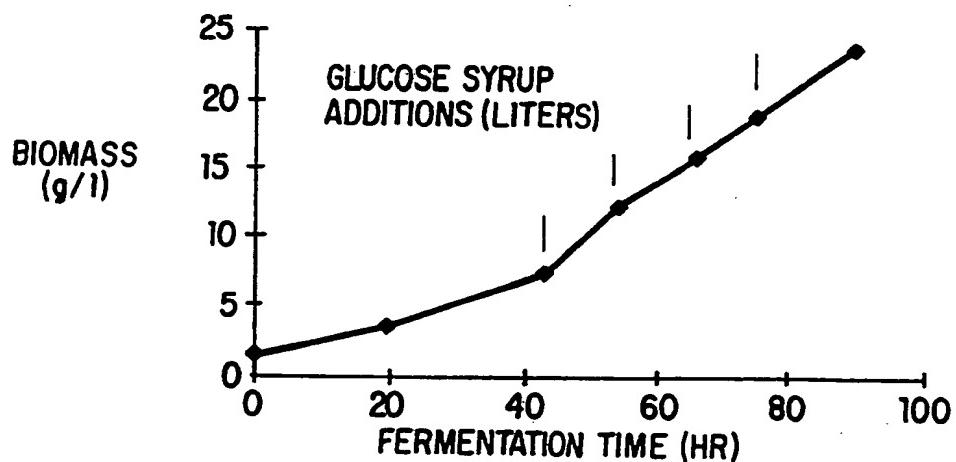
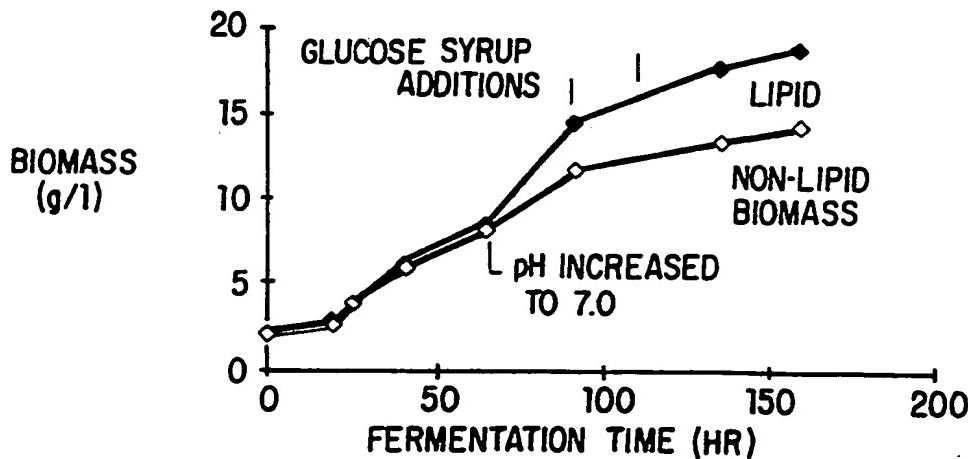


FIG. 2



SUBSTITUTE SHEET FIG. 3

## INTERNATIONAL SEARCH REPORT

PCT/US91/00733

International Application No.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 US: 426/2, 7, 61, 417, 442, 601, 635, 648, 805; 514/784, 789; 435/134, 136; 935/60, 66  
 IPC(5): A23L 1/054, 1/30; A61K 47/00 A23D 9/00, 9/02, 9/04; A23K 1/00, 1/18; C12P 7/64, 7/40

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

| Classification System   | Classification Symbols   |
|---|--|
| U.S.  | 426/2, 7, 61, 417, 442, 601, 635, 648, 805; 514/784, 789; 435/134, 136; 935/60, 66 |
| Documentation Searched other than Minimum Documentation<br>to the Extent that such Documents are Included in the Fields Searched <sup>5</sup> |  |

III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

| Category <sup>6</sup> | Citation of Document, <sup>10</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>   | Relevant to Claim No. <sup>18</sup> |
|-----------------------|--|-------------------------------------|
| V                     | US, A, 4,868,001 (MARUTA) 19 September 1989. see entire document.  | 33-34                               |
| X<br><del>Y,P</del>   | US, A, 4,960,795 (SALTE ET AL.) 02 OCTOBER 1990, see entire document.  | 35-38<br>35-38                      |
| V                     | US, A, 4,874,629 (CHANG ET AL.) 17 OCTOBER 1989, see entire document.  | 30-32                               |
| X<br><del>Y,P</del>   | US, A, 4,911,944 (HOLUB) 27 MARCH 1990. see entire document.   | 33-34<br>33-34                      |
| X<br><del>Y,F</del>   | Chemical Abstracts, Volume 114, No. 5. issued 04 February 1991, Noguchi et al., "Feeds Containing Highly Unsaturated Fatty Acids and Vitamin E for Swine and Production of Pork Using the Feeds", see page 630, column 1, abstract no. 41472w, Jpn. Kokai Tokkyo Koho JP 02,257,835. | 33-34<br>33-34                      |

\* Special categories of cited documents: <sup>15</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>2</sup>

10 April 1991

Date of Mailing of this International Search Report <sup>2</sup>

17 MAY 1991

International Searching Authority <sup>1</sup>Signature of Authorized Officer <sup>20</sup>

ISA/US

*Jeanne Marie*  
Jeanne Marie  
Jon P. Weber

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>   | Relevant to Claim No. 14               |
|------------|--|--|
| X/Y        | Chemical Abstracts, Volume 100, issued 1984, Kame et al., "Use of Fish Oil Fatty Acids (EPA and DHA) in Nutrition-Supplementing Foods and in Drugs", see page 472, column 2, abstract no. 66638d, Yushi, (1983), 36(12), 61-8.   | 53-54, 48-50, 45<br>45-48-50,<br>53-54 |
| X/Y        | BIOCHEMICA ET BIOPHYSICA ACTA. Volume 712, No. 3, issued 1982, U. Sonnenborn et al., "Purification and properties of the Fatty Acid Synthetase   | 1-32                                   |
|            | Complex from the Marine Dinoflagellate, Cryptocodinium cohnii", pages 523-534. See entire document, especially the Introduction and references cited therein.  | 1-32, 55                               |
| Y          | AMERICAN JOURNAL OF CLINICAL NUTRITION, Volume 51, No. 6, issued 1990, S.M. Innis et al., "Plasma and Red Blood Cell Fatty Acids of Low-Birth-Weight Infants Fed Their Mother's Expressed Breast Milk or Preterm-Infant Formula", pages 994-1000. See Abstract and Introduction. | 42-44,<br>47.51                        |
| X/Y        | EP, A, 0,231,904 (SCHWETKHARDT ET AL.) 30 January 1987. See entire document, especially mixture example, Table II, and claims 1 and 5.   | 42-44,<br>47.51<br>42-44, 47,<br>51    |
| Y, P       | US, A, 4,963,385 (ANTRIM ET AL.) 16 OCTOBER 1990, see especially columns one and two.  | 45, 53-54                              |

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET****V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers . . . . . because they relate to subject matter<sup>1</sup> not required to be searched by this Authority, namely:

2.  Claim numbers . . . . . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:

3.  Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>**

This International Searching Authority found multiple inventions in this international application as follows:

**See Attachment**

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. **Telephone practice**

2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.  As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.